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RESEARCH ARTICLE

GENETIC VARIABILITY OF *Passiflora* spp. BASED ON ISSR AND RAPD

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ABSTRACT

Passion fruit is an important crop in Brazil, therefore the study of its wild species is necessary to expand the passion flora genetic basis. This can be done by using the biotechnology that has enhanced the use of markers in improvement programs. During the germplasm characterization, molecular markers show different use complementing the morphological and agronomic information. This study aimed to characterize and to quantify the intra and interspecific genetic variability of *Passiflora* spp. accessions, using ISSR and RAPD molecular markers. 146 ISSR and 271 RAPD markers were obtained. They were used to estimate genetic dissimilarities among the accessions. The genetic dissimilarity matrices were used to perform cluster analysis by dendrogram using the Unweighted Pair-Group Method as grouping criterion and by graphic dispersion based on multidimensional scaling, using the principal coordinates method. There was a significant correlation of 0.56 between the genetic distances estimated based on ISSR and RAPD markers. The molecular markers characterization showed high genetic variability and adequate accessions differentiation. There was a clustering tendency of accessions and hybrids of the same species.

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INTRODUCTION

Genus *Passiflora* is considered as the most representative of the Passifloraceae family with nearly 400 species, most of which having its origin in the Tropical America. One hundred and thirty-nine of them are located all over the Brazilian territory making this country, specifically the center northern region, one of the main centers of the genus genetic diversity (BERNACCI *et al*., 2008; BERNACCI *et al*., 2013). The advanced modern biotechnology has enhanced the use of DNA markers in the routine of genetic improvement programs in their different stages and periods. For instance, during the germplasm characterization, molecular markers have shown different use complementing the morphological and agronomic information (FALEIRO *et al*., 2008). Bunphan *et al*. (2013) found that LW7 markers were effective when performing the assisted selection for the male sterility in sorghum. In order to exploit all the potential of wild species of passion fruit trees, a basic research in the characterization of genetic resources and a study targeting their improvement is necessary. To maximize the pre-improvement success, it is essential to have an integration between the phases and the activities and demands belonging to the improvement and post improvement program. In conclusion, the deep knowledge of an intra-specific genetic variability available for improvement is key for the process (FALEIRO *et al*., 2011). Therefore, the purpose of this study is to characterize and quantify the genetic variability of important *Passiflora* spp. Accessions using ISSR and RAPD molecular markers.

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MATERIAL AND METHODS

The present study was carried out using 15 *Passiflora* spp. accessions, including: 1. *P. alata* (CPAC MJ-02-17), 2. *P. nitida* (CPAC MJ-01-03), 3. *P. suberosa* (CPAC MJ-35-02), 4. *P. caerulea* (CPAC MJ-14-01), 5. *P. hatschbachii* (CPAC MJ-50-01), 6. *P. maliformis* (CPAC MJ-58-01), 7. *P. quadrangularis* x *P. alata* (CPAC MJ-H-44), 8. *P. sidifolia* (CPAC MJ-16-02), 9. *P. malacophylla* (CPAC MJ-43-01), 10. *P. alata* (CPAC MJ-02-09), 11. *P. alata* (CPAC MJ-02-19), 12. *P. quadrangularis* (CPAC MJ-07-03), 13. *P. cincinnata* (CPAC MJ-26-03), 14. *P. alata* BRS Mel do Cerrado and 15. *P. tenuifila* BRS Vita Fruit. The DNA extraction method was

Table 1. *Primers* tested and used to obtain ISSR and RAPD markers, for 15 *Passiflora* spp. accessions, sequence 5'→3' and the number of polymorphic bands (PB)

P. ISSR	Sequence 5'→3'	PB	P. RAPD	Sequence 5'→3'	PB
1-TriAAG3'RC	AAGAAGAAGAAGAAG	-	*1-OPD-07	TTGGCACGGG	27
2-TriACA3'RC	ACAACAACAACAACA	-	*2-OPD-10	GGTCTACACC	42
3-RriCAA3'RC	CAACAACAACAACAA	-	3-OPD-16	AGGGCGTAAG	-
4-TriAAC3'RC	AACAACAACAACAAC	-	*4-OPE-16	GGTGACTGTG	33
*5-TriAGC3'RC	AGCAGCAGCAGCAGC	26	5-OPE-18	GGACTGCAGA	-
6-TriAGG3'RC	AGGAGGAGGAGGAGG	-	*6-OPE-20	AACGGTGACC	45
*7-TriCAG3'RC	CAGCAGCAGCAGCAG	41	*7-OPF-01	ACGGATCCTG	32
*8-DiGA5'C	CGAGAGAGAGAGAGA	27	8-OPF-17	AACCCGGGAA	-
9-DiCA3'YG	CACACACACACACAC	-	9-OPG-01	CTACGGAGGA	-
10-DiCA5'CR	CACACACACACACAC	-	*10-OPG-05	CTGAGACGGA	33
11-DiGT3'YG	GTGTGTGTGTGTGTG	-	*11-OPG-08	TCACGTCCAC	22
12-DiCA3'G	CACACACACACACAC	-	12-OPH-04	GGAAGTCGCC	-
*13-DiGA3'C	GAGAGAGAGAGAGAG	24	13-OPH-16	TCTCAGCTGG	-
14-DiGA5'CY	AGAGAGAGAGAGAGA	-	*14-OPH-17	CACTCTCCTC	37
*15-DiGT5'CY	GTGTGTGTGTGTGTGT	28		Total	271
16-DiGA3'YC	GAGAGAGAGAGAGAG	-			
17-DiGA3'T	GAGAGAGAGAGAGAG	-			
18-DiCA3'RG	CACACACACACACAC	-			
Total		146			

Legend: P = *Primers*; *Selected and used *Primers* to produce ISSR and RAPD markers in 15 *Passiflora* spp accessions

The fresh vegetable tissue was macerated with a glass rod and 450 μL of a buffer containing 100 μM Tris-HCl (pH 8.3), 7% CTAB, 20 mM EDTA and 1.4 M NaCl were added to each sample The samples were then heated in water bath at 65 °C for 30 minutes. The desproteinization was performed by adding 400 μL of a chloroform solution: isoamyl alcohol (24:1). After that they were agitated to form an emulsion being thereafter centrifuged at 5.000 rpm for five minutes. Approximately, 200 μL of the supernatant were placed in 2 mL microtubes (type *Eppendorf*). Right after that, 200 μL of cold isopropanol (5 °C) were added to the supernatant, inverting the microtubes to promote the DNA precipitation. The tubes were then placed in the fridge for 30 minutes and then centrifuged at 7.000 rpm, for 10 minutes, removing the supernatant. The resulting *pellet* was washed for two times using 200 μL ethanol at 70% and dried at room temperature. After totally dried, the *pellet* was re-suspended in 100 μL Milli Q water, containing RNAse in a concentration equal to 40 μL mL^{-1}. The amount of DNA was estimated through the spectrophotometry at 260 ηm (A260) and the ratio A_{260} A_{280}^{-1} was used to evaluate the sample's purity and quality (SAMBROOCK; FRITSCH; MANIATS, 1989). The DNA samples of each accession were diluted to 5 ηg μL^{-1}. At first, 18 ISSR *primers* and 14 decamers RAPD *primers* were tested (Table 1). Amplification reactions for ISSR were performed (13 μL total volume) in 4.9 μL Milli Q water, 1.3 μL of buffer, 0.39 μL $MgCl_2$ (50 mM); 0.26 μL^1 deoxyribonucleotides (10 μM) (dATP, dTTP, dGTP and dCTP); 1.95 μL primer (2 μM) (Operon Technologies Inc., Alameda, CA, USA); 0.2 μL of Taq DNA polymerase (1 unity) and 3 μL of DNA (15 ηg). As for amplification reactions to obtain RAPD markers, they were performed in a 13 μL total volume by mixing 6.29 μL Milli Q water, 1.3 μL buffer 1x (Invitrogen), 0.78 μL $MgCl_2$ (50 mM); 0.13 μL desoxyribonucleotides (dATP, dTTP, dGTP e dCTP) (10 μM); 1.3 μL of a primer (2 μM) (Operon Technologies Inc., Alameda, CA, USA); 0.2 μL of Taq DNA polymerase (1 unity) and 3 μL DNA (15 ng).

Based on these tests, five *primers* were selected and used to obtain ISSR markers and eight to generate RAPD markers resulting in a greater quantity of polymorphic bands, showing a better quality of the amplifications (Table 1). The ISSR extraction was performed in a thermocycler, where the samples were at first denaturated at 94 ºC for 2 minutes, followed by 37 cycles, beginning with 15 seconds at 94 ºC; then followed by 30 seconds at 47 ºC and afterwards at 72 ºC for 1 minute. At the end of all cycles, the process was finalized during 7 minutes at 72 ºC, being cooled off at 4 ºC. On the other hand, the amplifications to extract RAPD markers were made in a thermocycler programmed for 40 cycles, each composed by the following sequence: 15 s at 94 ºC, 30 s at 35 ºC and 90 s at 72 ºC. After 40 cycles, a final extension period of six minutes at 72 ºC was conducted, and finally the temperature was decreased to 4 ºC. After being amplified, 3 μL of a mix containing bromophenol blue (0.25%) and glycerol (60%) in water were added to each sample. These samples were then submitted to an electrophoresis in agarose gel (1.2%), stained with ethidium bromide and embedded in a TBE buffer (Tris-Borate 90 mM, EDTA 1 mM). The electrophoretic separation lasted approximately 4 hours at 90 volts. After running the gels, they were photographed under ultraviolet light. ISSR and RAPD markers obtained were transformed in a binary matrix data to estimate genetic dissimilarity among the different genotypes based on the complement of the Nei and Li's similarity coefficient using Genes program (CRUZ, 2013). The genetic dissimilarity matrices were used to perform cluster analysis by dendrogram using the Unweighted Pair-Group Method as grouping criterion. The graphic dispersion was based on multidimensional scaling, using the principal coordinates method with the aids of SAS program (SAS INSTITUTE INC., 2008) and Statistica (STATSOFT INC., 2007). A descriptive analysis of the genetic distance estimates based on RAPD and ISSR markers (minimum and maximum values, averages and coefficient of variation) was performed and Pearson's correlations were calculated with the aids of Genes program (CRUZ, 2013).

RESULTS AND DISCUSSION

The analysis of 15 *Passiflora* spp. accessions using five *primers* resulted in a total of 146 ISSR markers with an

average of 29.2 markers by *primer*. The high percentage of polymorphic markers and average of markers by primers demonstrated also a high genetic variability among the accessions. This result might be explained by the amount of accessions used, because they are from different species and due to the efficacy of the ISSR technique in quantifying the *Passiflora* spp. variability. The distances ranged from 0.14 to 1.00. The maximum distance (1.00) estimated for ISSR markers was found between CPAC MJ-02-09 and CPAC MJ-43-01 accessions, CPAC MJ-07-03 and CPAC MJ-35-02 accessions, CPAC MJ-26-03 and CPAC MJ-02-09 accessions, and CPAC MJ-26-03 and CPAC MJ-02-19 accessions. The wide difference among the values for the genetic distance shows the accessions analysis with different degrees of dissimilarity, as it was also found by Pereira *et al*. (2015) using the ISSR marker. Cluster analysis and graphic dispersion proved the differences among the accessions (Figure 1A and B). Besides that, when considering as the cutoff point a 0.75 average genetic distance, five similarity groups were found: group one was composed by CPAC MJ-02-17, CPAC MJ-02-09, CPAC MJ-02-19, *P. alata* BRS Mel do Cerrado, CPAC MJ-07-03 and CPAC MJ-H-44 accessions, two composed by CPAC MJ-01-03 and *P. tenuifila* BRS Vita Fruit accessions, three included CPAC MJ-35-02 accessions, four included CPAC MJ-14-01, CPAC MJ-16-02, CPAC MJ-50-01 and CPAC MJ-58-01 accessions, and five composed by CPAC MJ-43-01 and CPAC MJ-26-03 accessions.

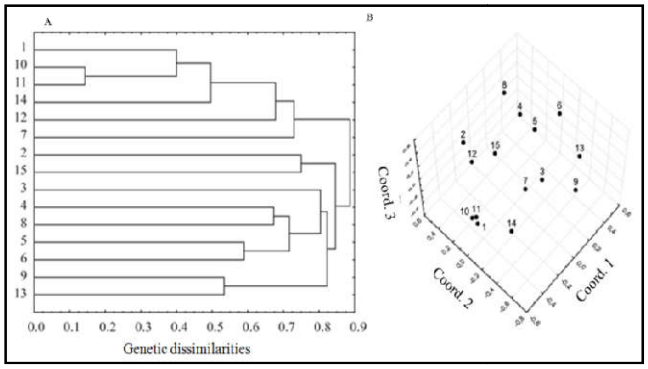

Figure 1. Cluster analysis (A) and graphic dispersion (B) of 15 *Passiflora* spp. accessions, based on the genetic dissimilarity matrix calculated using 146 ISSR markers. UPGMA method was used as a cluster criterion. The principal coordinate method was employed to perform the graphic dispersion analysis. The coefficient of cophenetic correlation (r) was 0.83. Legends: 1 - CPAC MJ-02-17, 2 - CPAC MJ-01-03, 3 - CPAC MJ-35-02, 4 - CPAC MJ-14-01, 5 - CPAC MJ-50-01, 6 - CPAC MJ-58-01, 7 - CPAC MJ-H-44, 8 - CPAC MJ-16-02, 9 - CPAC MJ-43-01, 10 - CPAC MJ-02-09, 11 - CPAC MJ-02-19, 12 - CPAC MJ-07-03, 13 - CPAC MJ-26-03, 14 - BRS Mel do Cerrado, 15. BRS Vita Fruit

In group 1, CPAC MJ-02-09 and CPAC MJ-02-19 accessions, formed a sub-group and CPAC MJ-02-17 was also close to this sub-group. In the sub-group that includes *P. alata* accessions, *P. alata* BRS Mel do Cerrado showed the greatest distance. Probably this small divergence related to all other *P. alata* accessions resulted from the improvement process to which this accession was submitted to. CPAC MJ-H-44 accession is a hybrid between *P. alata* and *P. quadrangularis* accessions. According to the analysis of ISSR markers, this accession was assigned to group 1 that includes all *P. alata* accessions and CPAC MJ-07-03 accessions, which was same species as their progenitors. Except for CPAC MJ-02-17, CPAC MJ-02-09 and CPAC MJ-02-19 accessions, all other accessions are widely distributed in the dispersion graphic (Figure 1B). The coefficient of cophenetic correlation by dendogram showed a good magnitude (r = 0.83) evidencing the consistency in the adjustment between the graphic representation of the genetic similarity and its original matrix, ensuring that inferences were made through a dendogram visual evaluation (Figure 1A). Based on the RAPD molecular marker technique analysis, 271 markers were obtained with 8 decamers *primers* totalizing an average of 33.89 markers by *primer*.

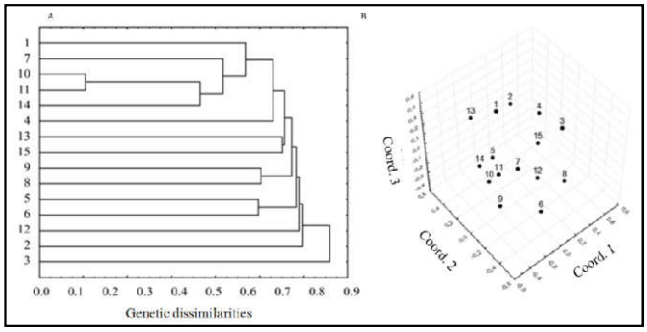

Figure 2. Cluster analysis (A) and graphic dispersion (B) of 15 *Passiflora* spp. accessions based on the genetic dissimilarity matrix using 271 RAPD markers. UPGMA was employed as a cluster criterion. The coefficient of cophenetic correlation (r) is 0.79. Legend: 1 - CPAC MJ-02-17, 2 - CPAC MJ-01-03, 3 - CPAC MJ-35-02, 4 - CPAC MJ-14-01, 5 - CPAC MJ-50-01, 6 - CPAC MJ-58-01, 7 - CPAC MJ-H-44, 8 - CPAC MJ-16-02, 9 - CPAC MJ-43-01, 10 - CPAC MJ-02-09, 11 - CPAC MJ-02-19, 12 - CPAC MJ-07-03, 13 - CPAC MJ-26-03, 14 - BRS Mel do Cerrado, 15. BRS Vita Fruit

Genetic distances generated by RAPD markers ranged from 0.21 to 0.85. When compared to ISSR technique, RAPD generated lower genetic distances estimates. The greatest dissimilarity was found between CPAC MJ-02-19 and CPAC MJ-35-02 accessions (0.85). Bellon *et al*. (2007) when studying the genetic variability of wild and commercial accessions of *Passiflora edulis* Sims. based on RAPD markers reported a high interspecific genetic variability in the *Passiflora* gender. The amplitude and genetic distance estimates among the accessions of different species are generally higher than the ones found for accessions of the same species. Cerqueira-Silva *et al*. (2010 a) researched *P. trintae*. and found out the distance varied from 0.07 and 0.42. Distances found in the present study have shown a higher variability than the one observed by Cerqueira-Silva *et al*. (2010 b) when they evaluated *P. edulis* and noticed a reduced variability among the accessions, which was from 0.12 to 0.19. CPAC MJ-35-02 and CPAC MJ-02-19 accessions were the most divergent among themselves (Figure 2A and B) which might be seen in the dendogram and dispersion graphic. Besides the difference found between these two accessions, the cluster analysis showed a big group of similarity composed by *P. alata* accessions, including CPAC MJ-02-17, CPAC MJ-H-44, CPAC MJ-02-09, CPAC MJ-02-19 and *P. alata* BRS Mel do Cerrado. Within this group, it could be concluded that CPAC MJ-02-09 and CPAC MJ-02-19 accessions are the closest. CPAC MJ-H-44, which is the result from the crossing between *P. alata* and *P. quadrangularis*, even being part of the biggest group showed to be closest to a sub-group composed

by CPAC MJ-02-09 and CPAC MJ-02-19 accessions than the group that included CPAC MJ-02-17 accession. All remaining accessions did not show similarity among themselves considering as the cutoff point a 0.58 average genetic distance. As it occurred with the ISSR markers, the coefficient of cophenetic correlation by dendogram had a good magnitude (r = 0.79) (Figure 2A). The results found by using two molecular techniques have clearly shown a wide genetic variability within the group of accessions that was studied, being a direct result of the genetic material analysis of several species of *Passiflora*. There was a clustering tendency of accessions of the same species or genealogy. This result was also found by Bellon *et al*. (2014) who used RAPD markers to study the recovering genome resulting from crossings programs involving the commercial species *P. edulis* and wild passion fruit species. The analysis of the Pearson correlation coefficient among the dissimilarity measurements calculated based on ISSR and RAPD markers demonstrated a positive (0.56) and highly significant correlation through the t test. Pires *et al*. (2015) when studying the genetic variability of *Dipteryx alata* Vog. and *Annona crassiflora* Mart. also found a positive and significant correlation between RAPD markers and microsatellites. These results evidenced the existing relationship and complementarity among these different molecular markers in studies regarding the genetic variability.

Both ISSR and RAPD markers have shown a wide genetic variability of *Passiflora* spp. accessions showing that ISSR and RAPD are good markers to be used to differentiate the accessions. This high evidenced genetic variability and the information obtained with ISSR and RAPD molecular markers are important for characterization programs, use of germplasm and genetic improvement of the passion fruit. This can be explained by the fact that the markers allow the complementation of morpho-characterization studies that will subside the selection of divergent genitors to form crossing and hybridization blocks to decrease the lost or narrowing of the genetic basis, maximizing the chances of obtaining desirable gene combinations.

Conclusion

The characterization based on ISSR and RAPD molecular markers has demonstrated a high genetic variability and differentiation of *Passiflora* spp. accessions evaluated in this work. There is a genetic structure among the evaluated accessions with a clustering tendency among accessions *P. alata* and materials resulting from the crossing involving accessions of this specie

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